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Morphology: still essential in a molecular world

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Abstract — Morphological characters have long served as the basis for mycological taxonomy. But with the advent of DNA sequence data, is morphology still useful? Will barcoding replace visual identification? Taxa in the *Dothideomycetes* serve to illustrate how molecular analyses have revised species relationships and higher-level systematics. *Aspergillus* species are now defined using a polyphasic approach with morphology assuming a lesser role. Sequence analyses likewise reveal that *Colletotrichum* species complexes once considered good morphological species now comprise many phylogenetically distinct species. Although *Phyllosticta* species concepts are less advanced, sequence data are expected to reveal new species in that genus as well. Molecularly supported higher taxa in *Dothideomycetes* often differ from those circumscribed by morphological characters. However, DNA barcodes, recently applauded as a magic formula for species identification, are yet to be determined for many genera, and too many GenBank sequences are wrongly named or contain sequencing errors. Thus, despite recent molecular advances, there is an unprecedented need for mycologists to return to the field, recollect species, and re-typify taxa with living cultures. Only after we obtain sequences from species and genera linked to properly named taxa will barcoding become successful.

Key words — anamorph, molecular phylogenetics, teleomorph, traditional taxonomy, typification

Introduction

Morphology has been the basis of nearly all fungal taxonomic studies. Numerous books and monographs use morphology alone to separate families, genera,

and species. Classical texts such as Marine Mycology, the Higher Fungi (Kohlmeyer & Kohlmeyer 1969), Genera of Hyphomycetes (Carmichael et al. 1980), and The Coelomycetes (Sutton 1980) are archetypal examples. Numerous important higher-level taxonomic texts have also been published using morphology for all class, ordinal, and familial placements. Texts such as A Re-evaluation of the Bitunicate Ascomycetes with Keys to Families and Genera (von Arx & Müller 1975) and Prodromus to Class *Loculoascomycetes* (Barr 1987) are classic examples.

Clearly morphology has underpinned taxonomic studies. In many other areas of fungal biology, it is essential to establish correct names and until recently there has been no way to identify a fungus without using morphological characters. Thus most fungal biochemistry, biotechnology, bioremediation, physiology, and plant pathology studies have cited species named after the fungi were identified through morphology (e.g. novel compounds — Evidente et al. 2008; chitinase production — Souza et al. 2003; bioremediation — Launen et al. 1995; physiology of *Colletotrichum graminicola* — Ali 1962; checklist of disease associated microorganisms in northern Australia — Hyde & Alcorn 1993). Similarly, most ecological studies relied on morphology to identify fungal communities (e.g. soil fungi communities — Ali-Shtayeh & Jamous 2000; fungal succession — Duong et al. 2008; endophytes — Hyde & Soytong 2008).

The situation however, is rapidly changing. Monographs of many genera now almost entirely rely on molecular data, and increasingly more often morphology is being replaced by molecular study (e.g. Tejesvi et al. 2007, Aveskamp et al. 2010). Ecological studies may now completely ignore morphology and fungal communities are identified through analysis of environmental DNA (Seena et al. 2008, Curlevski et al. 2010). The identities of fungi used in population genetics, biotechnology, and even biochemical studies are now often checked using sequence data only.

The results of these changes are rarely questioned, let alone discussed, yet most mycologists would agree that these changes should be advantageous. In this paper we explore *Aspergillus, Colletotrichum,* and *Phyllosticta,* genera where sequence data have to some extent profoundly affected species understanding. Below we discuss the effect of sequence data on understanding higher taxonomic levels in the *Dothideomycetes* and illustrate some unsolved problems in the new system. The aim is neither to criticize the studies nor to degrade the outcome, but to point out the resulting changes and confusion so that the mycological community can deliberate how best to manage such changes to everyone's benefit.

Phylogenetic methodology

Sequences were downloaded from GenBank and aligned using Clustal X. The alignment was optimized manually to allow maximum alignment and maximum sequence similarity. Gaps were treated as missing data. Phylogenetic analysis was carried out based on the aligned dataset by PAUP* 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded from all analyses. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed, and all multiple parsimonious trees were saved. Trees were figured in TreeView (Page 1996).

Discussion

Aspergillus, Colletotrichum and *Phyllosticta* – the process towards understanding a species

In many genera understanding what delimits a species has typically evolved from 1) a basic and relatively stable morphological concept (possibly including other characters such as cultural, growth rates, or mating), which often comprised species complexes, to 2) molecular revision where the morphological system starts to disintegrate and needs rethinking, and 3) a stabilized system based on molecular data with morphology taking a lesser role. Although eventually taxa may be identified solely using molecular data, in most genera this is decades away.

Aspergillus is advanced with respect to species delineation, mainly because it produces post harvest mycotoxins and valuable industrial chemicals (Geiser et al. 2007, Samson & Varga 2007). There has been a substantial increase in numbers of accepted taxa, with Rapier & Fennell (1965) recognizing 132 species, Geiser et al. (2008) estimating ~250 species, and Kirk et al. (2010) 266 species. Species delineation is based on a polyphasic approach with molecular data taking primary importance (Geiser et al. 2007). Multiple independent loci are now recommended when describing new species, particularly loci for which large datasets already exist, such as ITS, β-tubulin, calmodulin, actin, and RNA polymerase (Samson et al. 2007). All types are available in culture collections (Pitt & Samson 2000). Many species have now been sequenced for multiple genes and the understanding of species concepts in *Aspergillus* is advanced. Whole genomes have also been sequenced for at least eleven strains of nine species, with several others in the pipeline (Geiser et al. 2007; Samson, pers. comm.).

Sutton (1980) provided a practical key to 40 *Colletotrichum* species that provided a basic species identification text. Although often difficult to decide whether to key a fungus to one or another species, the key was convenient and

descriptions brief. Even after 27 years and >4000 *Colletotrichum* publications, Sutton's text served as a necessary and convenient tool for placing names on taxa. The first molecular data on *Colletotrichum* were published after 1990 (e.g. Bailey et al. 1996, Correll et al. 1993, Fabre et al. 1995); although the results were revealing, the data began to complicate species identification (Hyde et al. 2009a, b). There was, however, no attempt to stabilize species concepts in a formal way, so that sequences deposited in GenBank were unknowingly often wrongly named. Not until 2007–2008 were several *Colletotrichum* species epitypified (Shenoy et al. 2007, Cannon et al. 2008), thereby enabling comparisons of reference sequence data against data from fresh collections. This commenced the period of reconciling *Colletotrichum* species, especially in the difficult complexes. Recent studies have introduced 15 new species (most in the "gloeosporioides" species complex), epitypification of 14 *Colletotrichum* species, and generation of sequence data for ex-type cultures of 46 species (Hyde et al. 2009b; Damm et al. 2009; Prihastuti et al. 2009, 2010; Shivas & Yu 2009; Phoulivong et al. 2010; Yang et al. 2009, 2010; Wikee et al. 2011).

Figure 1 provides an example of the confusion that molecular data can produce. We generated the phylogram by downloading 41 GenBank ITS sequences, of which 25 were labeled *Colletotrichum gloeosporioides*. In Fig. 1 *C. gloeosporioides* epitype sequences cluster at the top of the tree, while clades containing putative *C. gloeosporioides* strains — some representing very distantly related species —are scattered throughout, illustrating the diversity of one species name in GenBank. Cai et al. (2009a) have estimated that >86% of the *C. gloeosporioides* names in GenBank considerably diverge from the epitype and are likely to represent other *Colletotrichum* species. As *C. gloeosporioides* represents a species complex comprising numerous diverse species, great care must be used when downloading sequences labeled as 'gloeosporioides' from GenBank. Ultimately, only sequence data from the epitype strain should be used to characterize the species.

Compared with *Aspergillus* and *Colletotrichum*, understanding *Guignardia* and its *Phyllosticta* anamorphs is less advanced. *Guignardia* comprises 335 records (Index Fungorum) and has no monograph, although species from various hosts have been reviewed (e.g. palms — Hyde 1995; *Podocarpus* — Crous et al. 1996). Van der Aa & Vanev (2002) accepted 141 species based on cultural and morphological characteristics in their monograph on *Phyllosticta*. As very few living types appear to exist in these genera, Wulandari et al. (2009) compared their new species causing tan spot of pomelo in Asia with many questionably labeled *Phyllosticta* sequences from GenBank. D.M. Lam & N. Wulandari (unpublished) also sequenced many *Guignardia* and *Phyllosticta* strains from CBS, but as few represented type strains, their conclusions were limited and may never be published. There is a need to designate epitypes

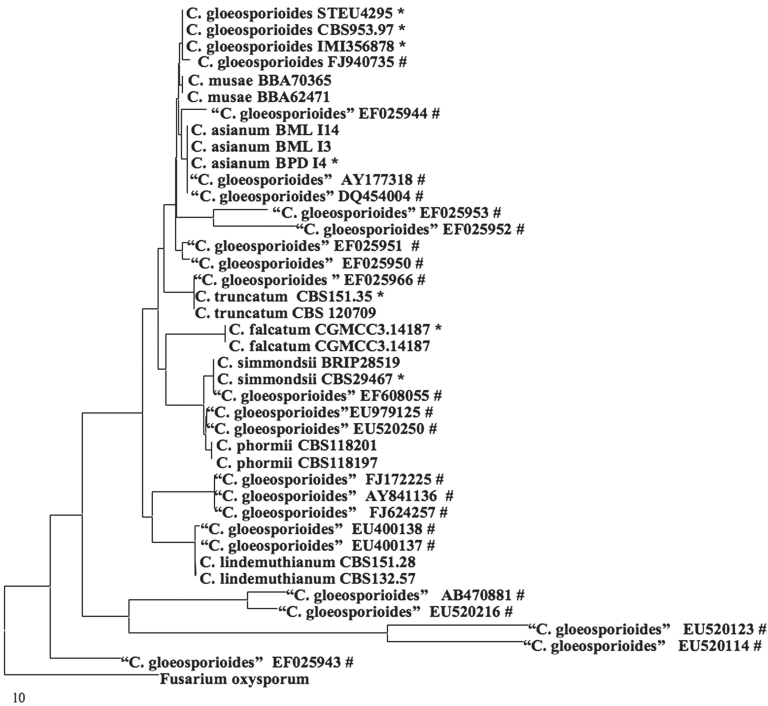

Fig. 1. Maximum parsimony phylogram generated from ITS sequence analysis of "*Colletotrichum gloeosporioides*" downloaded from GenBank with other related taxa. Data were analysed with random addition sequence, unweighted parsimony, and treating gaps as missing data. # indicates ITS sequences of "*Colletotrichum gloeosporioides*" downloaded from GenBank; * indicates sequences derived from ex-type cultures.

for species of *Phyllosticta* and the teleomorph *Guignardia*, so that a clear understanding of the status of species and their biological relationships can be obtained.

Guignardia mangiferae A.J. Roy offers a second example of confusion resulting from molecular data (Fig 2). This name has been extensively applied to an endophyte isolated by Rodrigues et al. (2004); many putative *G. mangiferae* strains were used by Wulandari et al. (2009). However, no type of *G. mangiferae* can be found (Wulandari, pers comm.) nor has it ever been epitypified. Thus this recent name has been used arbitrarily for endophytic strains producing obtrullate ascospores. The obtrullate ascospore type, however, can be found in numerous species (e.g. *G. eucalyptorum* Crous, *G. smilacis* A.J. Roy, *G. graminea* Lobik) and most likely comprises a species complex that could

have a much older name. In Fig. 2 we downloaded a selection of *G. mangiferae* labeled strains from GenBank to illustrate the diversity the name represents. It is therefore unwise to name a *Guignardia* or *Phyllosticta* species based solely on sequence similarity with a GenBank sequence.

The above examples serve to illustrate how molecular data can resolve species understanding in some plant pathogenic genera yet pose challenges in interpretation. We should remember that many previous studies likely applied incorrect names to their organisms. Type cultures must be sequenced, and where no such cultures exist, fresh collections are needed. Both type cultures and fresh collections should be fully characterized using morphology, sequence analyses, and other polyphasic approaches. Only by using such methods can we begin to understand genera and their individual species complexes. Such understanding now exists for *Aspergillus* and *Penicillium*, is advanced in *Fusarium*, is progressing in *Colletotrichum,* and has only begun in *Guignardia*/*Phyllosticta* and *Pestalotiopsis.* The simple message is that although molecular data may eventually identify taxa in these genera, an enormous concerted effort is needed to recollect, morphologically characterize, epitypify, sequence, analyze, and combine all data with other polyphasic characters before we will make any real progress in understanding species in these important genera. It is also suggested that NCBI should rename an entry if there are sufficient evidences supporting to do so.

The *Loculoascomycetes*

AFTOL (All Fungi Tree of Life) aimed to find natural classifications for fungi based on multi-locus phylogeny, rather than visual, relationships (Schoch et al. 2006). The project made considerable progress towards understanding fungi at the higher levels, particularly in the basidiomycetes. Classes of fungi are similarly better resolved in the ascomycetes, although the *Dothideomycetes* offer a good example where molecular analyses have resulted in uncertainty, especially at the family level.

The issue of Studies in Mycology (Schoch et al. 2009) devoted to the *Dothideomycetes* resolved many problems at the higher taxonomic levels (order, family) but may have created more confusion than intended. What classical mycologists such as J.A. von Arx, E. Müller, and M.E. Barr previously considered to be orders and families and the characters they used to diagnose such (von Arx & Müller 1975; Barr 1987) are, in many cases, no longer usable. Unfortunately, although molecular data can place taxa at the family and in some cases generic levels, there has been little effort made in attempting to correlate phylogeny with phenotypes (Suetrong et al. 2009, Zhang et al. 2009a).

For example, the *Lophiostomataceae* and *Trematosphaeriaceae* cluster as separate families and contain elements that can be linked by very few characters

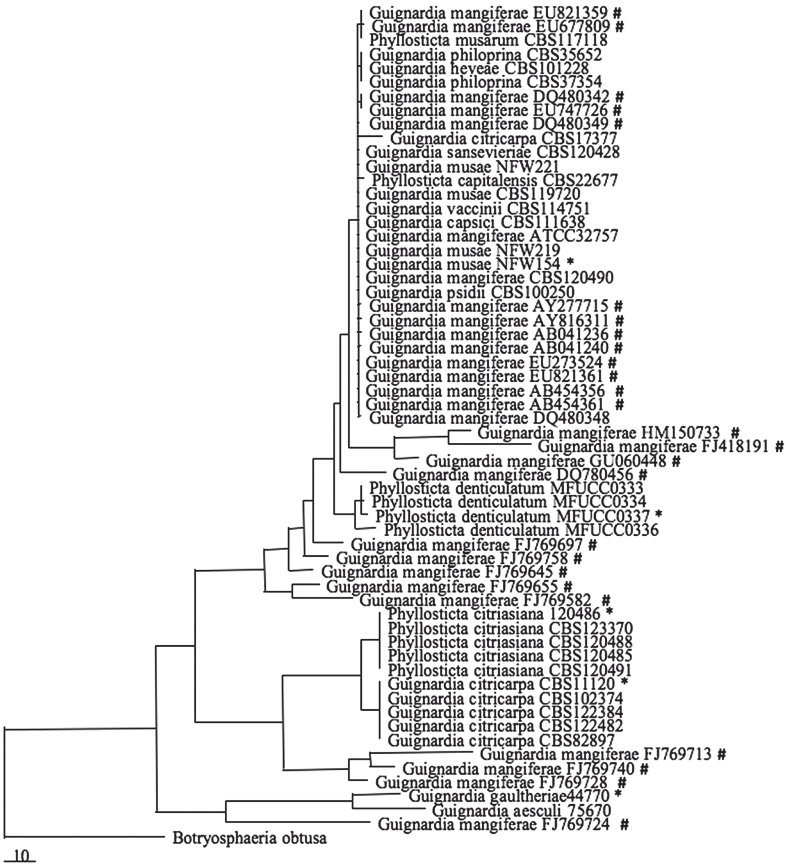

Fig. 2. Maximum parsimony phylogram generated from ITS sequence analysis of "*Guignardia mangiferae*" downloaded from GenBank with other related taxa. Data were analysed with random addition sequence, unweighted parsimony, and treating gaps as missing data. # indicates ITS sequences of "*Guignardia mangiferae*" downloaded from GenBank; * indicates sequences derived from ex-type cultures.

— the same characters found in other families. The *Lophiostomataceae* include *Lophiostoma,* some species placed in *Thyridaria,* and a new genus *Misturatosphaeria* (Mugambi & Huhndorf 2009; Zhang et al. 2009a,b). *Lophiostoma* species are characterized by ascomata that are erumpent with slot- or slit-like ostioles and may have raised flanges (Holm & Holm 1988), while in *Misturatosphaeria* ascomata are erumpent to superficial with often raised rounded apices and ascospores are phragmosporous or dictyosporous (Mugambi & Huhndorf 2009). Dictyosporous ascospore types are found throughout the

Dothideomycetes but not — until now — within *Lophiostomataceae*. At the moment, there is a distinct lack of defining characters that can be used for this family. Mugambi & Huhndorf (2009) themselves state, "despite morphological differences of *Misturatosphaeria* from other lophiostomataceous fungi, we feel justified in placing it in *Lophiostomataceae* at this point due to the strong support received in their analysis."

Tetraplosphaeriaceae (Tanaka et al. 2009) is basal to most families in *Pleosporales* and yet previous classification systems would have probably placed the species in *Astrosphaeriella* (Hyde et al. 2000). The main distinguishing characters of the family are the *Tetraploa*-like anamorphs; however the ascomata (immersed or superficial), pseudoparaphyses (cellular or trabecular), and ascospore (fusiform to cylindrical, 1–3-septate, hyaline or pale brown) forms are found throughout the *Dothideomycetes*. Therefore if a researcher encounters the teleomorph stage only, it would be difficult to use morphology to place the taxon, even at the family level, unless the characters are identical to an existing species in the literature.

In other groups in the *Dothideomycetes* there are so few sequences available that phylograms reveal very little information concerning the species at any level. This is true of taxa in the *Capnodiaceae* and *Microthyriaceae* and in numerous genera (e.g *Muyocopron*, *Trichodelitschia*) (see Boehm et al. 2009, Schoch et al. 2009).

What is the way forward? Many sequences used in the issue of Studies in Mycology on the *Dothideomycetes* are linked to cultures from poorly documented taxa while only a few are linked to type material. This will create doubt in the minds of readers because generic types must be used in such analyses. Again, a concerted effort is needed to recollect, document characters, isolate, and deposit herbarium materials and/or living cultures. In this way we will have accurately documented morphological characters that are linked to sequence data of accurately named species; only then can we confidently start to understand relationships in *Dothideomycetes* and be confident in the conclusions arising from combined morphological and molecular classifications.

Linking anamorphs to teleomorphs

There has been much expectation amongst mycologists that molecular analyses of anamorphic fungi will be able to link them to teleomorphs or at least provide an idea of their positions in the *Ascomycota* (Shenoy et al. 2006, 2007). Several studies have shown that morphological characters traditionally used to delimit anamorphic fungi are less informative in inferring fungal phylogenies. For example, in traditional taxonomy morphologically well-defined genera such as *Chalara* and *Sporidesmium* appear to be highly polyphasic (Shenoy et al. 2006, Cai et al. 2009b). Re-evaluation of the evolutionary significance of anamorphic

characters should therefore be carried out to 'rebuild' morphological classification. Morphology will then once again become important for identifying species, provided type specimens and derived cultures have been used in the reconstruction. If unavailable, the fungus should be interpreted by a freshly collected material from original hosts and localities, accurate documentation, isolation, sequencing, and deposition in herbaria as epitypes with living ex-type cultures. Only in this way will an accurate understanding of the natural placement of anamorphs in the teleomorphic scheme be achieved.

Barcoding and GenBank difficulties and solutions

There are important initiatives to barcode the fungi (Santamaria et al. 2009, Seifert 2009). However, we feel that the benefit gained from large scale sequencing of fungal isolates will be diluted if sequence data from too few properly named taxa or types are deposited in public databases. As illustrated by Figs 1–2, the lack of sequences with reliably applied names in public databases would make barcoding currently unworkable. This deficiency must be corrected at the same time as barcoding takes place. As the type specimens and derived type cultures are not always available, there needs to be a concerted effort by mycologists to go back to the field and recollect the fungi. Taxonomic experts must carefully name those fungi and where possible designate epitypes with derived living cultures. Once we obtain sequences from species and genera that are linked to properly characterized taxa, we can really start to understand the fungi. Only then will barcoding work. These approaches will be useful in a few fungal studies as data obtained from molecular analysis of environmental samples, linking of anamorphs and teleomorphs, and the proper naming of species in biochemistry, pathology, and biotechnology research publications become precise.

Concluding remarks

Fungal systematics has irreversibly stepped into the phylogenetics era. Molecular diagnosis through barcoding is favored by most researchers because it seemingly provides an easy and quick assessment of the fungus at hand and does not require years of training. This, however, does not exclude morphology from modern systematics, as morphological characters are the most easily accessible. The characters used to define species, genera, families, and orders nonetheless need reevaluation in light of sequence generated phylogenetic relationships. Morphological characters would then be used in agreement with new classification schemes and thus correspond to the natural phylogeny. The success of molecular diagnosis and barcoding, however, largely depends on comparing sequence data from type specimens. Most fungal names lack living type specimens and cannot be sequenced. There is consequently an

urgent need to epitypify all such fungi and deposit living ex-type cultures and derived sequence data in public culture collections and databases. Mycologists must go back to field and recollect important species and generic types and re-characterize these taxa using a polyphasic approach. Incorporating morphology is essential for establishing species concepts and higher taxonomic frameworks. Until much more data has been generated from types and many more accurately named species are deposited in public databases, confusion will remain. To eliminate the confusion, morphology is not only not outdated but is a necessity.

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